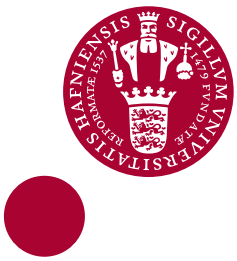

A collaborative European exercise on mRNA-based body fluid/skin typing and interpretation of DNA and RNA results

van den Berge, M; Carracedo, A; Gomes, I; Graham, E A M; Haas, C; Hjort, Benjamin Benn; Hoff-Olsen, P; Maroñas, O; Mevåg, B; Morling, N; Niederstätter, H; Parson, W; Schneider, P M; Court, D Syndercombe; Vidaki, A; Sijen, T

Published in:
Forensic science international. Genetics

DOI:
10.1016/j.fsigen.2014.01.006

Publication date:
2014

Forensic Science International: Genetics 10 (2014) 40–48

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

A collaborative European exercise on mRNA-based body fluid/skin typing and interpretation of DNA and RNA results

M. van den Berge [a], A. Carracedo [b,c], I. Gomes [d], E.A.M. Graham [e], C. Haas [f], B. Hjort [g], P. Hoff-Olsen [h], O. Maroñas [b], B. Mevåg [h], N. Morling [g], H. Niederstätter [i], W. Parson [i,j], P.M. Schneider [d], D. Syndercombe Court [k], A. Vidaki [k], T. Sijen [a,*]

[a] Netherlands Forensic Institute, The Hague, The Netherlands
[b] Forensic Genetics Unit, Institute of Forensic Science, Genomic Medicine Group, University of Santiago de Compostela, Spain
[c] Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia
[d] Institute of Legal Medicine, Medical Faculty, University of Cologne, Germany
[e] Northumbria University Centre for Forensic Science, Newcastle, UK
[f] Institute of Legal Medicine, University of Zurich, Switzerland
[g] Section of Forensic Genetics, Department of Forensic Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[h] Department of Forensic Genetics, Norwegian Institute of Public Health, Oslo, Norway
[i] Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria
[j] Eberly College of Science, Penn State University, University Park, PA, USA
[k] Department of Forensic and Analytical Science, King's College London, London, UK

ARTICLE INFO

Article history:
Received 10 December 2013
Received in revised form 17 January 2014
Accepted 19 January 2014

Keywords:
Forensic science
Body fluid identification
mRNA profiling
RNA interpretation
EUROFORGEN exercise

ABSTRACT

The European Forensic Genetics Network of Excellence (EUROFORGEN-NoE) undertook a collaborative project on mRNA-based body fluid/skin typing and the interpretation of the resulting RNA and DNA data. Although both body fluids and skin are composed of a variety of cell types with different functions and gene expression profiles, we refer to the procedure as 'cell type inference'. Nine laboratories participated in the project and used a 20-marker multiplex to analyse samples that were centrally prepared and thoroughly tested prior to shipment. Specimens of increasing complexity were assessed that ranged from reference PCR products, cDNAs of indicated or unnamed cell type source(s), to challenging mock casework stains. From this specimen set, information on the overall sensitivity and specificity of the various markers was obtained. In addition, the reliability of a scoring system for inference of cell types was assessed. This scoring system builds on replicate RNA analyses and the ratio observed/possible peaks for each cell type [1]. The results of the exercise support the usefulness of this scoring system. When interpreting the data obtained from the analysis of the mock casework stains, the participating laboratories were asked to integrate the DNA and RNA results and associate donor and cell type where possible. A large variation for the integrated interpretations of the DNA and RNA data was obtained including correct interpretations. We infer that with expertise in analysing RNA profiles, clear guidelines for data interpretation and awareness regarding potential pitfalls in associating donors and cell types, mRNA-based cell type inference can be implemented for forensic casework.

1. Introduction

The potential of mRNA profiling to infer which body fluid or tissue resides in an evidentiary sample has been well demonstrated in the past decade. The identification of specific, sensitive and robust markers, poses a continuous search that is undertaken for a growing number of body fluids and organs [2–23]. Evaluation of the performance of these markers is well assisted by large collaborative exercises that have been performed for several body fluids [24–27]. Suitable markers have been combined in end-point reverse transcription PCR (RT-PCR) systems that assess the presence of multiple cell types simultaneously [28–31]. Most of these assays carry multiple markers per cell type, as expression of individual mRNAs varies with biological function and for individuals or physiological condition. As a result, RNA profiles appear much less balanced than DNA profiles and sometimes

* Corresponding author at: Department of Human Biological Traces, Netherlands Forensic Institute, Laan van Ypenburg 6, The Hague 2497GB, The Netherlands. Tel.: +31 70 8886666; fax: +31 70 8886555.
E-mail address: t.sijen@nfi.minvenj.nl (T. Sijen).

1872-4973/$ – see front matter © 2014 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsigen.2014.01.006

marker dropout occurs. Marker drop-in may arise from the presence of non-specific transcripts in a cell due to back ground level gene expression or as a result of spurious transcription that occurs whenever RNA polymerase binds to DNA. Variation in RNA profiling data may be further stimulated by the use of a rather high number of amplification cycles (33 in [30], 35 in [24–27], 30 in [29], 30 or 35 depending on the body fluid in [31]), and stochastic amplification effects [33] are seen when replicate RNA analyses are performed [32]. These issues have triggered the development of interpretation strategies for RNA profiles that allow for some marker absence and spurious signals [1,31]. The scoring methodologies have limitations with body fluids that express markers of other body fluids as well and cannot discriminate co-expressed cell types (e.g. blood co-expressed in menstrual secretion) from a mixture of the two cell types (e.g. peripheral blood and menstrual secretion). Mixed stains are challenging to interpret anyhow as one donor may give multiple cell types or multiple donors the same cell type. Combined interpretation of RNA and DNA profiling results may be only possible when gender-specific body fluids and donors of different genders are involved, as peak height-based association of donor and cell type was found to be risky even in straightforward two donor–two cell type mixtures [32].

A collaborative exercise was organised among the partners of the European Forensic Genetics Network of Excellence (EUROFORGEN-NoE) in order to assess the value of mRNA analysis in a forensic context. An RNA analysis system was taken that is routinely used for casework at the Netherlands Forensic Institute (NFI), and each participant was provided with the same set of specimens that had increasing complexity. The most complex samples were mock casework stains for which laboratories not only scored the RNA results but also provided a forensic interpretation, building on the estimated number and genders of contributors, the cell types regarded present and, if possible, association of donor and cell types. As these stains were designed to be challenging and complex, potential pitfalls for integrated DNA and RNA data interpretation become apparent.

2. Materials and methods

2.1. Samples and materials provided

The mRNA profiling exercise was divided into two parts: part 1 included purified PCR products and cDNA specimens and part 2 comprised mock casework stains. Details are provided in Table 1. Specimens were prepared at the organising laboratory (NFI) with informed consent of the voluntary donors whose cell material was used. Saliva and semen were collected in tube, blood through a finger prick (Accu-chek, Softclix Pro, Roche Diagnostics GmbH, Germany), vaginal mucosa on cotton swab, menstrual secretion on Viba brushes (Rovers, Oss, the Netherlands) and skin by rubbing textiles over the face. Textiles used as substrates for stains were freed from contaminating DNA by irradiating each side with 254 nm UV light in a CL-1000 UV CrossLinker (UVP, Upland, USA) at 900 mJ/cm^2 for 30 min. For stain 1, equal amounts of saliva of two donors were mixed and 40 μL were added to cotton swabs. For stain 2, two Viba brushes with menstrual secretion (day 2 menstrual cycle) were gently shaken in 500 μL PBS (phosphate buffered saline) for 0.5 h after which 200 μL whole blood were added and 5 μL of the mixture were transferred to pieces of fleece. Since the menstrual secretion signals were surpassing those of blood, 50 μL neat blood were added on top of the stain. For stain 3, skin donor 1 rubbed one side and skin donor 2 the other side of a patch of linen over the face. Then, spots of 1 μL 100-fold diluted blood of one of these donors were placed on the linen and small areas of cloth around these blood spots were excised. For stain 4, nail clippings of relatively short nails having contact with skin were collected and a fresh vaginal mucosa swab was rubbed over the nail clippings to transfer cell material. After drying, 1 μL seminal fluid of an azoospermic male was added. All specimens and reagents sent to participating laboratories were thoroughly tested prior to shipment. When shipping part 1 reagents, an amount of multiplex primer mix sufficient for both exercise parts was included so that all results were obtained using a single batch of primer mix. Part 1 reagents and specimens were sent on dry ice taking 1–7 days, when in part 2 only dried stains were sent normal mail was used. When additional reagents such as extraction chemistry were requested dry ice was used. These shipments took between 3 and 9 days.

2.2. DNA/RNA extraction, DNA quantification, reverse transcription

At the organising laboratory, samples were subjected to DNA/RNA co-extraction, quantification of DNA extracts, DNase treatment of RNA extracts and reverse transcription (both plus RT reaction and minus RT control) of 10 μL RNA aliquots as described in [30]. In each extraction, a negative and positive control (water or

Table 1
Overview of the samples and objectives assessed during the mRNA profiling exercise.

Part	Samples	Tasks and objectives
1	Purified[a] PCR products, cell type indicated	Adjust provided bin sets
	7 samples: each cell type[b] and blank	CE sensitivity differences for laboratories
	Single source cDNAs, cell type indicated	One PCR per sample with 1 μL cDNA input
	24 samples: 3 inputs[c] and minus RT for each cell type	Overall marker sensitivity and frequency of marker drop-in
	cDNAs with unspecified cell type	Two PCRs per sample: 0.5 μL and 2.0 μL input
	10 samples: communicated as single source, but including one	Familiarisation serial cDNA input approach
	mixture and one water sample	Overall performance: marker drop-ins and marker dropouts
	Mixed cDNAs, unspecified cell types	Determine optimal cDNA input from 0.5 μL and 2.0 μL tests
	4 samples: mixtures of two or three body fluids	Generate four informative replicates for scoring RNA results
		Overall usefulness of scoring system
2A	Complex stains, unspecified cell types	Extraction, DNA/RNA profiling and questionnaire
	4 samples: on a variety of substrates	DNA: estimated minimum number of contributors and genders
		RNA: scoring peaks and cell types
		Interpretation: cell types present and association donor/cell type
2B	NFI dataset for the four complex stains	Compare interpretations for same dataset
	1 DNA profile and 4 replicate RNA profiles	

[a] Purification by MinElute columns using a low salt strength buffer to elute products. Prior to shipment it was tested that the fluorescently labelled PCR products are stable in this buffer, which is not the case if purified into water.
[b] Six cell types are included: blood, saliva, semen, vaginal mucosa, menstrual secretion and skin.
[c] cDNAs were derived from three amounts of RNA.

5 μL blood spotted on FTA) was included. Negative controls did not show signals, positive controls showed correct peaks (housekeeping and blood markers only). For preparation of cDNA specimens, an appropriate number of cDNA batches (20 μL each) were pooled and redistributed into aliquots of 5–20 μL. All protocols were provided as example protocols, but participants could use chemistries and instrumentations of choice. An overview of the methodologies used during stain analysis is presented in Supplementary Table 1. Participating laboratories were asked to use the entire stain to prevent differences from using less material. For low level samples (indicated by a DNA yield below 2 ng), it was advised to concentrate the RNA extract down to 12 μL by applying an ethanol precipitation as described in [30] and use 10 μL in a single plus RT cDNA reaction and 2 μL in a minus RT cDNA control.

2.3. Endpoint PCR

The 19-plex described in [30] was supplemented with vaginal mucosa marker CYP2B7P1 [19] (amplicons size 146 bp; forward primer 5′-VIC-AGTCTACCAGGGATATGGCATG; reverse primer 5′-CTATCAGACACTGAGCCTCGTCC; final primer concentration 1.6 μM), menstrual secretion marker MMP10 [5,27] (amplicons size 107 bp; forward primer 5′-VIC-GCATCTTGCATTCCTTGTG-CTGTTG; reverse primer 5′-GGTATTGCTGGGCAAGATCCTTGTT; final primer concentration 1.6 μM) and skin marker LCE1C [16,18] (amplicons size 99 bp; forward primer 5′-NED-TGTGACCC-CGCTCCTGAATCCG; reverse primer 5′-CTTGGGAGGGCACTTGG-GGGTG; final primer concentration 0.02 μM). To create the space in the multiplex to add these three markers, the general mucosa markers KRT13 and SPRR2A were removed. In this way, a 20-plex was created. A large batch of 5× primer stock for this multiplex was prepared and aliquoted to provide all laboratories with the exact same primer mixture in all RT-PCRs.

The suggested cDNA inputs in the RT-PCR analyses were 1 μL for cDNA specimens with indicated cell type, 0.5 and 2 μL for cDNA samples with unspecified cell type(s), 0.1, 0.5, 1 and 4 μL for stain cDNAs and 3.5 μL for minus RT, negative and positive controls.

2.4. Capillary electrophoresis and analysis of DNA and RNA profiles

Amplified fragments were separated and detected on various types of standard genetic analysers using different separation matrices as indicated in Supplementary Table 1. As RT-PCR products are generated using a homemade multiplex, removal of dye-blobs prior to analysis is essential, and the various approaches used are indicated in Supplementary Table 1. For the analysis of RNA profiles, a 150 rfu detection threshold was suggested by the organising laboratory [30]. However, some labs used a lower threshold of 50 or 100 rfu as indicated in Supplementary Table 1. No intervention occurred because peaks were just below or above threshold. For the analysis of DNA profiles, participating laboratories used their own protocols.

2.5. Scoring of RNA data

Following the procedure described in [1], four replicate RNA profiles were generated using an informative cDNA input, and the RNA data were evaluated by applying an '$x = n/2$' scoring system per cell type [1]. Here, 'x' reflects the number of observed and 'n' the number of theoretically possible peaks in all replicates. A cell type is scored as 'observed' if $x \geq n/2$, 'not observed' if $x = 0$ and 'sporadically observed' if $0 < x < n/2$. For co-expressed cell types, 'and fits' is added when (sporadically) observed. Cell types scored as 'sporadically observed' are generally regarded as 'not reliably observed' and tissues scored as 'and fits' as 'not present as such' [1].

3. Results

3.1. Analysis of cDNAs

Nine laboratories including the organising laboratory participated in the exercise. All laboratories had experience with RNA analyses; eight had participated (one as organiser) in EDNAP RNA exercises [24–27]. The exercise started with the analysis of reference RT-PCR, which enabled adjustment of the provided marker bin settings in the analysis software if necessary. Some differences in peak heights occurred; one participant had on average 2.5 times higher peaks than the organising laboratory while another had approximately 0.5 times lower peaks, which seems due to the use of lower injection settings (Supplementary Table 1).

The next set consisted of 24 cDNA specimens derived from three RNA inputs and a minus RT control for each of the six cell types. The results are presented in Table 2 and differences in marker sensitivity are evident. Although this finding may have been affected by the use of a single donation for each cell type, the trends befit observations in the organising laboratory. The difference is most extreme for the vaginal mucosa markers: whilst CYP2B7P1 responds almost fully for the three RNA inputs, no peaks are obtained for HBD1 and only some for MUC4. For HBD1 this probably derives from suboptimal performance in multiplex analysis [30]. For the housekeeping markers, GAPDH appears to be the least and 18S-rRNA the most robust marker. Non-specific signals are occasionally seen both in non-target cell types and in minus RT samples by all laboratories. Three cases appear more frequent: (1) blood and especially CD93 signals for vaginal mucosa, which is unprecedented and may have been the result of a trace of blood (or menstrual secretion for which CD93 appears the most prominent blood marker) in this specific donation; (2) skin marker signals (LOR and to lesser extent CDSN) for vaginal mucosa, indicating that LCE1C is the more specific skin marker and (3) MMP10 signals for various non-target cell types and minus RT blanks, which may be related to the relatively high signals for true MMP10 peaks, which are on average 5320 rfu, while for the other markers the average height ranges from 495 rfu (MUC4) to 3190 rfu (HBB). A lower primer concentration for MMP10 may be beneficial.

The next task involved the analysis of ten numbered cDNA specimens, indicated to be single source but purposefully comprising eight single source cDNAs (blood, saliva, two times skin, vaginal mucosa, menstrual secretion, semen fertile donor, semen azoospermic male), one mixture (vaginal mucosa with blood) and one water sample. To illustrate the effect of cDNA input on RNA profiling results, participants were asked to generate two RNA profiles with a four-fold difference in cDNA input (0.5 and 2.0 μL, respectively). In theory, the participants can detect a total of 208 cell type-specific peaks for the ten cDNA specimens with each cDNA input. Using the 0.5 μL input, 109 peaks were detected and with the higher input (2 μL except for one laboratory that used 1 μL), 161 peaks were observed. Marker dropout was predominantly seen for vaginal mucosa marker HBD1 and blood marker AMICA1, which are both among the less sensitive markers when testing the RNA input series (Table 2). Peaks detected using both the 0.5 μL and the 2 μL input (91 in total) are on average 3.9 times higher for the 2 μL, which complies with the four-fold increased input. With a higher amount of cDNA, more non-target peaks were seen as well: 21 for the lower and 42 for the higher input, which is a total of 63 marker drop-ins in 140 RNA profiles. Again, skin markers LOR and CDSN gave false positive signals in vaginal mucosa and menstrual secretion specimens (23 and 16 observations for LOR and CDSN respectively), and menstrual marker MMP10 showed occasional drop-in signals (13 times) among all

Table 2
Percentages of detected markers when analysing cDNAs derived from three inputs of single source RNAs. For each cDNA specimen, one RNA profile was produced by each participant with an input of 1 μL cDNA (Table 1).

		Blood[a]			Saliva[a]			Semen[a]			Skin[a]			Menst secr[a]			Vag muc[a]			-RT[b]
RNA input (μL)		1.0	0.4	0.2	1.0	0.4	0.2	1.0	0.4	0.2	1.0	0.4	0.2	1.0	0.25	0.125	1.0	0.4	0.2	-
Cell type specific markers																				
Blood	HBB	100%[c]	88%	63%										13%	0%	0%				
	CD93	100%	63%	13%							13%			100%	50%	50%	50%	38%	13%	2%
	AMICA1	63%	25%	13%										38%	0%	0%	13%			
Saliva	STATH				88%	13%	13%													
	HTN3				75%	38%	38%													
Semen	SEMG1						25%	100%	100%	100%										
	PRM1							88%	88%	88%										
Skin	CDSN				13%						100%	100%	63%	13%	13%	13%	63%	25%	13%	
	LCE1C							13%			88%	38%	38%							
	LOR										100%	63%	0%	13%			88%	88%	63%	2%
Menst secr	MMP10	13%				13%	13%	13%			13%		13%	100%	100%	88%	13%			10%
	MMP7				13%									100%	63%	25%				
	MMP11													88%	38%	25%				
Vag muc	HBD1										13%			0%	0%	0%	0%	0%	0%	
	MUC4										13%			100%	75%	38%	25%	0%	0%	
	CYP2B7P1													100%	100%	38%	100%	100%	88%	2%
General mucosa marker																				
	KRT4				88%	63%	38%			13%				100%	100%	75%	100%	88%	88%	
Housekeeping markers																				
	ACTB	100%	100%	88%	25%	0%	13%	38%	50%	13%	88%	38%	13%	100%	100%	100%	100%	100%	100%	
	18S-rRNA	100%	100%	88%	63%	38%	38%	75%	63%	50%	88%	88%	88%	100%	100%	100%	100%	88%	63%	
	GAPDH	100%	88%	38%	13%	0%	0%	25%	13%	0%	25%	13%	13%	100%	88%	63%	88%	63%	25%	

[a]Results are based on the data of eight laboratories as one laboratory used an unsuccessful method to purify PCR products.
[b]Six minus RT samples (one per cell type) were provided to each participant and percentages are based on 48 profiles.
[c]Correct marker signals are shaded green, false positive signals are shaded red. Darker shades indicate that a higher percentage of laboratories observe the signal.

specimens including the water sample (data not shown). The marker calling for the mixture and the water sample was not affected by labelling them as single source cDNAs.

The last set of cDNAs involved four mixtures having two or three cell types in balanced (1:1, based on the corresponding DNA profiles) or unbalanced (up to 1:10) ratios. Participants were asked to determine optimal cDNA input from the profiling results for a 0.5 and 2 μL input and generate four replicate RNA profiles for application of the '$x = n/2$' scoring system. The results are presented in Table 3. It becomes apparent that it is helpful to use the category 'sporadically observed' (which we generally regard as not observed) as this category was used 21 times for cell types not present in the mixtures and nine times for cell types present. In total, a cell type was missed eleven times, which in seven events concerned skin being the lowest component in mixture four (ratio 10:5:1) (Table 3). The other four missed inferences relate to three different components in three mixtures (Table 3). A false positive skin identification was obtained by all participants in the third mixture. This seems a consequence of the unintended responses of LOR and lesser extent CDSN in vaginal mucosa: in the 32 RNA profiles generated by the eight participants for this mixture, 29 LOR, 18 CDSN and no LCE1C signals were seen. It seems beneficial to remove LOR from the multiplex or exclude LOR results from interpretation by the scoring system.

3.2. Analysis of stains

Exercise part 1 on the cDNA sets familiarised participants with RNA profile analysis using the provided 20-plex and the application of the '$x = n/2$' counting system. The results were provided as feedback prior to the second exercise part that involved DNA and RNA analysis of challenging mock casework stains. The design of the four stains is given in Table 4. The participants were asked to estimate the number and genders of the donors, derive a result score for each cell type using to the '$x = n/2$' counting system and determine which cell types are present or not present (or 'no statement'). Both LOR and HBD1 were excluded during scoring because of non-specific signals with vaginal mucosa or insufficient multiplex amplification. Participants used various methodologies to extract nucleic acids and derive DNA and RNA profiles (Supplementary Table 1), and yields and profiling outcomes showed considerable variation (Table 5, Supplementary Figure 1).

For stain 1 (two females giving saliva), all participants scored saliva, which is the only body fluid present, as observed. Four times a cell type not present in the stain was scored as observed: once blood, once menstrual secretion and twice skin. Two of these false positives (blood and once skin) relate to the use of a lower detection threshold than that of 150 rfu advised by the organising laboratory. The false menstrual secretion score relates to the analysis of slightly overloaded RNA profiles in which trailing signals occur about ten nucleotides before the parent peaks: the trailing signal of saliva marker HTN3 fits the bin of the MMP7 menstrual secretion marker (although 0.3 nt smaller than true MMP7 peaks), and together with MMP10 background signals (also seen in part 1, Table 2) menstrual secretion gets scored 'observed'. Actually all peaks including those for housekeeping markers show these trailing signals and with more expertise in analysing RNA

Table 3
Results of the analysis of four cDNA mixtures. Participants generated four informative RNA profiles and applied '$x = n/2$' counting system that scores results into five different categories [1].

Blood & Saliva (1:1)	Blood[a]	Saliva	Semen	Menstrual	Vaginal	Skin	Mucosa
Not observed			8/8[b]	6/8	6/8	7/8	
Observed	8/8	8/8					
Sporadically observed				2/8	2/8	1/8	
Observed & fits							8/8
Sporadically observed & fits							
Blood & Saliva & Skin (1:1:1)	**Blood**	**Saliva**	**Semen**	**Menstrual**	**Vaginal**	**Skin**	**Mucosa**
Not observed			8/8	3/8	5/8		
Observed	8/8	6/8				8/8	
Sporadically observed		2/8		5/8	3/8		
Observed & fits							8/8
Sporadically observed & fits							
Vaginal & Semen (fertile) (10:1)	**Blood**	**Saliva**	**Semen**	**Menstrual**	**Vaginal**	**Skin**	**Mucosa**
Not observed	6/8	8/8		2/8			
Observed			7/8		8/8	8/8	
Sporadically observed	2/8		1/8	6/8			
Observed & fits							8/8
Sporadically observed & fits							
Menstrual & Semen (sterile) & Skin (10:5:1)	**Blood**	**Saliva**	**Semen**	**Menstrual**	**Vaginal**	**Skin**	**Mucosa**
Not observed		8/8				2/8	
Observed	1/8		7/8	8/8	2/8	1/8	
Sporadically observed	1/8		1/8			5/8	
Observed & fits	3/8				6/8		8/8
Sporadically observed & fits	3/8						

[a]Colour coding for cell types is: black cell = present; white cell = not present; grey cell = co-expressed.
[b]Colour coding of table cells for the results is: green cell = correct i.e. not observed when not present or observed (& fits) when present; light green cell = sporadically observed (regarded not observed) when not present; red cell = incorrect i.e. observed but not present or not observed when present; light red cell = sporadically observed (regarded not observed) but present; grey cell = aberrant result for co-expressed cell types.

Table 4
Design of the four stains that compose part 2 of the exercise.

Substrate	Preparation	Challenge
1: Cotton swab	Saliva ♀$_{D1}$ & saliva ♀$_{D2}$: daughter & mother; unequal DNA amounts	One body fluid by two donors; Parent–child relation between donors
2: Coloured fleece	Skin ♀$_{D1}$ & menstrual secr. ♀$_{D1}$ & blood ♂$_{D2}$	Presence blood masked by menstrual secretion; Skin and menstrual secretion given by same donor
3: Green linen	Skin ♀$_{D1}$ & diluted blood ♀$_{D1}$ & skin ♀$_{D2}$	Low amount: EtOH precipitation may be needed; Donor 1 gives two cell types, skin given by two donors
4: Nail clipping	Nail/skin ♂$_{D1}$ & vaginal muc. ♀$_{D2}$ & semen azoospermic male ♂$_{D3}$	Only two of three donors will contribute DNA; Male DNA part represents skin and not seminal fluid

Table 5
Summarised results of the DNA and RNA data for the four stains (Table 4) that compose part two of the exercise.

Feature	Scoring[a]	Stain 1	Stain 2	Stain 3	Stain 4	All stains
Estimated minimum number and genders contributors	One	♀: 1/8	-	♀: 5/8	♀: 4/8	-
	Two	♀&♀: 7/8	♀&♂: 8/8	♀&♀: 3/8	♀&♂: 2/8 ♀&?: 2/8	-
Average[b] % detected non-shared alleles	Donor 1	99%	100%	99%	16%	-
	Donor 2	84%	86%	28%	100%[c]	-
Cell types present	Observed[de]	8/8	19/24[f]	6/16	18/24	71% (51/72)
	Not observed	0/8	1/24	5/16	2/24	11% (8/72)
	Sporadically obs[d]	0/8	4/24	5/16	4/24	13% (13/72)
Cell types not present	Observed	4/40	0/16	0/32	0/24	4% (4/112)
	Not observed	25/40	15/16	27/32	17/24	75% (84/112)
	Sporadically obs	11/40	1/16	5/32	7/24	21% (24/112)

[a]Data of eight laboratories are included; for one laboratory the replicates seem contaminated by multiple cell types.
[b]No standard deviation is provided; results per participant are given in Supplementary Figure 1.
[c]For donor 3 no specific alleles were observed.
[d]Both the categories 'observed' and 'observed & fits' or 'sporadically observed' and 'sporadically observed & fits'. For more details see Supplementary Table 1.
[e]Colour coding of table cells for the results is: green cell = correct i.e. observed when present or not observed or sporadically observed when not present; red cell = incorrect i.e. not observed or sporadically observed when present or observed but not present.
[f]Only the added cell types, not co-expressed cell types are regarded (so skin, blood and menstrual but not vaginal).

profiles generated with the 20-marker multiplex, these false signals may have been recognised. The fourth false positive observation (skin) seems to relate to the analysis of slightly over-amplified profiles on a 3500 genetic analyser. The dynamic rfu range of the 3500 platform is approximately four-fold higher than that of the 3130XL platform that was used to generate the provided analytical thresholds. Concomitantly, true signals had up to 34,000 rfu while the false skin signals were between 800 and

Table 6
Data (bold) and interpretations (not bold) for the NFI dataset on the four stains regarding DNA (A), RNA (B) and combined results (C).

A	Quant (ng/μL)[a]		AMEL (rfu)		Number alleles[b]		Donor 1[b]		Donor 2[b]		Interpretation DNA results
	Total	Male	X	Y	Profile	Locus	Alleles	Rfu	Alleles	Rfu	
Stain 1: $♀_{D1\ child}$ & $♀_{D2\ parent}$	1.02	0.0	1651	–	36	≤3	10/10	509	10/11	95	4x two ♀; 2x ♀ & unknown; 1x one ♀
Stain 2: $♀_{D1}$ & $♂_{D2}$	1.23	0.12	1719	345	52	≤4	26/26	620	26/26	576	7x ♀ & ♂
Stain 3: $♀_{D1\ major}$ & $♀_{D2\ minor}$	0.02	0.0	1348	–	35	≤4	17/17	523	6/19	75	3x two ♀; 2x ♀ & unknown; 2x one ♀
Stain 4: $♂_{D1\ minor}$ & $♀_{D2\ major}$ & $♂_{D3\ sterile}$	0.54	0.01	2391	–	30	≤4	22/22	1621	3/23	113	3x ♀ & ♂; 1x ♀ & unknown; 3x one ♀

B	Blood	Saliva	Semen	Skin	Menstrual	Vaginal
Stain 1:	–	Saliva	–	–	–	–
Marker count	0/12	8/8	0/8	0/8	0/12	0/8
Result score[c]	7x not obs	7x obs	7x not obs	7x not obs	7x not obs	7x not obs
Presence score	7x not present	7x present	7x not present	7x not present	7x not present	7x not present
Stain 2:	Blood	–	–	Skin	Menstrual	–
Marker count	8/12	0/8	0/8	4/8	12/12	0/8
Result score	4x obs; 3x obs&fits	7x not obs	7x not obs	6x obs; 1x spor&fits	7x obs	7x not obs
Presence score	4x present; 1x not present; 2x no statement	7x not present	7x not present	6x present; 1x no statement[d]	7x present	7x not present
Stain 3:	Blood	–	–	Skin	–	–
Marker count	11/12	0/8	0/8	8/8	3/12	0/8
Result score	7x obs	7x not obs	7x not obs	7x obs	6x: spor; 1x not obs[e]	6x: not obs; 1x spor[e]
Presence score	7x present	7x not present	7x not present	7x present	2x not present; 5x no statement	7x not present
Stain 4:	–	–	Semen (sterile)	Skin (nail)	–	–
Marker count	2/12	0/8	4/8	8/8	0/12	6/8
Result score	6x spor; 1x spor&fits[f]	7x not obs	5x obs; 1x spor[g]; 1x spor&fits[h]	6x obs; 1x spor&fits[i]	7x not obs	6x obs; 1x not obs[j]
Presence score	2x no statement; 5x not present	7x not present	6x present (3x sterile); 1x no statement	7x present	7x not present	6x present; 1x not present

C	Overall interpretation	Correctness
Stain 1: $♀_{D1\ child}{}^{saliva}$ & $♀_{D2\ parent}{}^{saliva}$		
1x	$♀_{major}$ = saliva, $♀_{minor}$ = saliva	Correct
3x	$♀_{major}$ = saliva, $♀_{minor}$ = saliva or unknown cell type	Correct
1x	$♀_{major}$ = saliva, presence 2nd donor not confirmed	Correct although one donor missed
2x	Two ♀ donors and saliva, no association	Correct; some under-interpretation
Stain 2: $♀_{D1}{}^{skin\ \&\ menstrual}$ & $♂_{D2}{}^{blood}$		
1x	♀ = menstrual, ♂ = skin	Incorrect; presence blood missed
2x	♀ = menstrual, ♂ = skin or blood; ♀ = menstrual, ♂ = probably skin	Correct; little over-interpretation
2x	♀ = menstrual, ♂ = skin and/or blood; ♀ = ms, ♂ no assoc.	Correct
1x	♀ = menstrual, skin no statement, blood either ♀ or ♂ donor	Correct; unclear formulation
1x	♀ & ♂ DNA donor, menstrual & skin (blood co-expressed), no association	Incorrect; presence blood missed
Stain 3: $♀_{D1\ major}{}^{skin\ \&\ diluted\ blood}$ & $♀_{D2\ minor}{}^{skin}$		
4x	Two $♀_{DNA}$ donors, blood & skin, no association	Correct
1x	$♀_{major}$ = skin, $♀_{minor}$ = blood or $♀_{D1}$ = skin + blood, $♀_{D2}$ = unknown	Correct; little over-interpretation
1x	Confident on one ♀ donor, blood & skin will be from her	Incomplete for DNA, risky on association
1x	♀ donor and skin present, possibly 2nd ♀ giving blood or sporadic menstrual with blood co-expressed, no association	Correct; but it is missed that 2nd donor could have given skin
Stain 4: $♂_{D1\ minor}{}^{skin\ (nail)}$ & $♀_{D2\ major}{}^{vaginal}$ & $♂_{D3\ azoospermic}{}^{semen}$		
2x	$♀_{major}$ = vaginal + (probably) skin, $♂_{minor}$ = semen	Incorrect; male donor did not give semen
1x	$♀_{major}$ = vaginal, $♂_{minor}$ = semen, skin no statement	Incorrect; male donor did not give semen
2x	One ♀ donor = vaginal + skin, semen not reliable or only RNA	Incorrect; male donor not provide skin
1x	♀ and unknown, skin & vaginal & seminal fluid, no association	Under-interpretation; 2nd donor is ♂ (low male quant) so♀ gave vaginal
1x	No report: discrepancy observing semen while no ♂ genotype	Incorrect; no PRM1/spermatozoa/DNA

[a] Sensitivity Alu-based DNA quantification system is >0.0005 ng/μL for total and >0.004 ng/μL for male human DNA concentration.
[b] Allele counts on 15 STRs, amelogenin excluded. Donor-specific counts involve only non-shared alleles with homozygous alleles counted as two.
[c] Observed is abbreviated obs, sporadically observed as spor. Results of 7 participants: one did not return data and the organiser was aware of stain design.
[d] Laboratory 4: skin signals may be a false positive response (3 CDSN and 1 LCE1C peaks) with menstrual/vaginal.
[e] Laboratory 1: scoring for menstrual and vaginal swapped?
[f] Laboratory 7: the 2 blood peaks are sporadic and may fit vaginal.
[g] Laboratory 8: 4/8 semen peaks scored as 'sporadically observed', and interpreted as 'no statement'.
[h] Laboratory 1: 4/8 semen peaks scored as 'sporadically observed & fits', interpreted as 'present': mis-scoring?
[i] Laboratory 1: 8/8 skin peaks scored as 'sporadically observed & fits', interpreted as 'present': mis-scoring?
[j] Laboratory 1: 6/8 vaginal peaks scored as 'not observed', interpreted as 'not present': mis-scoring?

2400 rfu. When transferring RNA profiling to 3500 platforms, analytical thresholds need to be re-established.

For stains 2, 3 and 4, no false positive scorings occur (Table 5). However, for these three stains not all cell types present were scored as observed. For stain 2 (female giving menstrual secretion and skin plus male giving blood), all participants observed menstrual secretion, while blood was scored as observed by six and skin by five of the eight laboratories. This is striking for blood as the blood donor corresponds to almost half of the total rfu weight in most of the DNA profiles. Apparently, blood gives relatively low RNA signals compared to the DNA signals or menstrual secretion gives relatively low DNA signals compared to the RNA signals indicating, as already shown in [32], that it is not appropriate to associate the strongest signals from RNA and DNA typing of co-extracted samples with each other. Stain 3 (skin and diluted blood from one female plus skin from another female) was challenging due to the low amounts of cell material present, which is reflected by a low number of observed scorings for present cell types: blood is scored only once and skin five times as observed (Table 5). Only two of the participants proceeded to ethanol precipitation of the RNA prior to cDNA synthesis, and the one positive scoring for blood was obtained by one of these participants. For stain 4 (nail clipping from a male with vaginal mucosa female and seminal fluid azoospermic male), skin and vaginal mucosa were scored as observed by six laboratories while semen was reported as observed four times. Since the semen contribution is of an azoospermic male, only the seminal fluid marker SEMG1 will respond. To have semen scored as 'observed', SEMG1 needs to give a positive signal in all four replicates. Actually, this was only the case for three of the four laboratories: one participant had three (true) SEMG1 signals and one (false) PRM1 peak that was a pull-up from the LOR signal, which could have been recognised by peak shape and size as it was 0.9 nucleotide larger than a true PRM1 signal. When considering the scorings of all stains together, 4% were false positives scores that can be explained from suboptimal profile analysis and 11% were false negative scores (Table 5). The category 'sporadically observed (& fits)' was used for 13% of the scorings for cell types present and for 21% of the scorings for cell types not present, suggesting that this is a useful category as it seems to lower the number of false positive results.

3.3. Combined interpretation of DNA and RNA data for the stains

Using the inferences on donor numbers, their genders and the cell types observed, participants were requested to give a combined interpretation of DNA and RNA data. However, the underlying profiling data were so different that it was not constructive to compare these interpretations, and the dataset of the organising laboratory was sent out. This set consisted of the DNA quantification results (total and male-specific), one DNA profile and four RNA replicate profiles for each stain. Characteristics of this dataset are summarised in Table 6. The participants estimated the number and genders of the donors, derived a result score for each cell type and determined which cell types are present or not present, or receive 'no statement'. With stains 1, 3 and 4 participants provided different responses for the numbers and genders of donors (Table 6), indicating that the exact same DNA data are evaluated differently. The cell type scorings are mainly in accordance with the provided guidelines (Table 6). In all cases when cell types were scored as not observed, participants indicated these cell types as not present. Cell types scored as observed were always regarded present. Blood in stain 2 was classified four times as observed and three times as observed and fits because menstrual secretion signals were detected as well. In all instances when 'observed and fits' was chosen, 'no statement' was selected for presence. The sporadic signals seen for menstrual secretion in stain 3 and blood in stain 4 received different interpretations; six times the corresponding cell types were regarded as not present and six times 'no statement' was made.

The interpretations for the DNA and RNA data were combined into a verbal conclusion in which donors and cell types were associated, if possible. For stains 1, 2 and 3 the majority of the verbal conclusions are correct, have a correct interpretation among the multiple options given in the statement, or leave room for the correct alternative by using 'probably' (Table 6). For stain 2 twice an incorrect interpretation was given that appears to derive from not recognising that in case menstrual secretion is present blood signals may also originate from a peripheral blood contribution. For stain 4, which was clearly the most complex stain that was sent out, no correct interpretation was provided. In this stain, the presence of the second male donor giving seminal fluid is fully masked at the DNA level by the female giving vaginal mucosa and the first male providing the nail clipping. Sterile seminal fluid does not carry spermatozoa, but low amounts of epithelial cells may be present and provide some DNA. Actually when a Y-STR profile was generated, all alleles of the nail donor and some low level signals for the seminal fluid donor were visible (results not shown). Most of the incorrect interpretations associated the male DNA component with the semen contribution without realising that this male may have provided the skin residing on the nail clipping and another male the seminal fluid. Other participants underestimated the number of contributors and consequently linked both vaginal mucosa and skin to the female donor, while she only provided vaginal mucosa. One participant reported a discrepancy for the lack of a male profile while a positive result for semen was obtained, which implies that the azoospermic status of the donor was not recognised. It is also noticed that some participants were very reluctant to make associations such as for stain 1 for which one participant did not link the major DNA contributor to saliva (Table 6). Interestingly, sometimes participants mentioned that DNA signals may derive from unknown cell types or cell types 'below detection', while they had indicated that the not observed cell types were all 'not present'. Apparently, 'not present' is rather used as 'seemingly not present, but there may be signals below detection' and terms like 'no indication for presence' or 'the presence can not be excluded' may be more appropriate.

4. Discussion and concluding remark

This collaborative EUROFORGEN-NoE exercise explored the usefulness of forensic cell type inference by mRNA profiling. The methodology would expedite from a good human RNA quantitation system. The exercise used a 20-marker multiplex in which most markers performed acceptably except skin marker LOR that showed cross-reactivity with vaginal mucosa and vaginal mucosa marker HBD1 that had low amplification success. These markers are best removed or replaced in an updated future multiplex. The addition of a second seminal fluid marker would increase the detection chance of semen from azoospermic males since now, a signal is needed in all four replicates to confirm presence. Furthermore, the primer concentrations for menstrual secretion marker MMP10 can be lowered to prevent background signals and over-amplification. Redesign of the multiplex is challenging because of multiplex spacing and marker balance. RNA amplicons are preferably sized between 70 and 150 bp to allow analysis of compromised samples. Consequently, limited space is available for markers and recurrent bleed-through and artefact signals (split peaks and trailing signals) that should not culminate in the bins of other markers. Primer concentrations need to be such that both good sensitivity and low noise levels are obtained. Furthermore, the use of a relatively high detection threshold appears beneficial to prevent false positive marker callings. Experience with the

multiplex may aid profile analysis as artefact peaks are better recognised.

Even for an optimised multiplex, it may be inevitable that RNA profiles have signal imbalances, marker dropout and marker drop-in, as mRNA expression is influenced by various biological factors. To assess the validity of cell type signals, interpretation guidelines are applied. In this study, we used the '$x = n/2$' scoring system [1]. This system worked adequately as only few false positive scores were obtained (Table 5), which were predominantly due to signal calls on artefact peaks. Employing these guidelines may come at the cost of not inferring all cell types that are present, which is important to keep in mind during case interpretation. As an alternative approach, a numerical scoring method is described [31] in which values are assigned to each of the used mRNA markers (five per body fluid) based on correct and incorrect expression in samples of known origin. From these numerical values a body fluid score is calculated and positive body fluid identification is given when the combined marker value is higher than a pre-determined threshold value. This is clearly different from the '$x = n/2$' scoring system, in which all markers have the same weight, implying that all markers are evenly effective for cell type inference. This is not always the case as for instance seminal fluid and spermatozoa markers respond different when analysing semen of an azoospermic male (which could be compensated by adding a second seminal fluid marker or by scoring presence of seminal fluid upon regarding SEMG1 results only). On the other hand, the '$x = n/2$' method presents a general approach applicable to different mRNA profiling assays like cell and organ typing [21,30]. A comparative study including compromised samples would be informative to assess both interpretation strategies.

In a forensic case, DNA and RNA data need to be combined. Distribution of a DNA/RNA dataset derived for four truly challenging stains assessed this aspect. These stains covered the most prominent complications in DNA/RNA profiling such as same cell type given by multiple donors (stains 1 and 3), same donor giving multiple cell types (stains 2 and 3), masking of a cell type (blood, stain 2) by a co-expressing cell type (menstrual secretion), low level analysis (stain 3) and a cell type giving RNA but no (or hardly) DNA signals (seminal fluid azoospermic male, stain 4). No helpful context information such as 'the nails were clipped from person X' or reference profiles were given. In addition, not all participants were experienced with formulating forensic verbal statements. While for stains 1, 2 and 3 many correct interpretations were given, none of the interpretations for stain 4 were correct. This stain represents the forensic analysis of nail clippings taken from an assailant of digital penetration of a victim who had had previous intercourse with an azoospermic male. Although this is an unusual scenario, it may happen and serves to illustrate that awareness regarding such interpretation pitfalls is important when proceeding to RNA analysis in forensic casework.

In conclusion, with expertise in analysing RNA profiles, clear guidelines for data interpretation and awareness regarding potential interpretation pitfalls mRNA-based cell type inference may be ready for implementation in forensic casework.

Acknowledgements

The work leading to these results was financially supported from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 285487 (EUROFORGEN-NoE). Daniela Niederwieser (Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria) and Gnanagowry Shanthan (Department of Forensic Genetics, Norwegian Institute of Public Health, Oslo, Norway) are acknowledged for their excellent technical assistance. We thank Corina Benschop (Netherlands Forensic Institute, The Hague, The Netherlands) for critically reading the manuscript and Petra Maaskant, Ingrid Blom and Ankie van Gorp (Netherlands Forensic Institute, The Hague, The Netherlands) for testing the NFI dataset for interpretation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.01.006.

References

[1] A. Lindenbergh, P. Maaskant, T. Sijen, Implementation of RNA profiling in forensic casework, Forensic Sci. Int. Genet. 7 (2012) 159–166.
[2] M. Bauer, A. Kraus, D. Patzelt, Detection of epithelial cells in dried blood stains by reverse transcriptase-polymerase chain reaction, J. Forensic Sci. 44 (1999) 1232–1236.
[3] M. Bauer, D. Patzelt, Protamine mRNA as molecular marker for spermatozoa in semen stains, Int. J. Legal Med. 117 (2003) 175–179.
[4] J. Juusola, J. Ballantyne, Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification, Forensic Sci. Int. 135 (2003) 85–96.
[5] J. Juusola, J. Ballantyne, mRNA profiling for body fluid identification by multiplex quantitative RT-PCR, J. Forensic Sci. 52 (2007) 1252–1262.
[6] M. Bauer, D. Patzelt, Identification of menstrual blood by real time RT-PCR: technical improvements and the practical value of negative test results, Forensic Sci. Int. 174 (2008) 55–59.
[7] M. Setzer, J. Juusola, J. Ballantyne, Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains, J. Forensic Sci. 53 (2008) 296–305.
[8] D. Zubakov, E. Hanekamp, M. Kokshoorn, W. van Ijcken, M. Kayser, Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of timewise degraded samples, Int. J. Legal Med. 122 (2008) 135–142.
[9] C. Haas, B. Klesser, C. Maake, W. Bar, A. Kratzer, mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR, Forensic Sci. Int. Genet. 3 (2009) 80–88.
[10] C. Haas, C. Muheim, A. Kratzer, W. Bär, C. Maake, mRNA profiling for the identification of sperm and seminal plasma, Forensic Sci. Int. Genet. Suppl. Ser. 2 (2009) 534–535.
[11] K. Sakurada, H. Ikegaya, H. Fukushima, T. Akutsu, K. Watanabe, M. Yoshino, Evaluation of mRNA-based approach for identification of saliva and semen, Legal Med. (Tokyo) 11 (2009) 125–128.
[12] R.I. Fleming, S. Harbison, The use of bacteria for the identification of vaginal secretions, Forensic Sci. Int. Genet. 4 (2010) 311–315.
[13] K. Sakurada, T. Akutsu, H. Fukushima, K. Watanabe, M. Yoshino, Detection of dermcidin for sweat identification by real-time RT-PCR and ELISA, Forensic Sci. Int. 194 (2010) 80–84.
[14] C. Haas, E. Hanson, A. Kratzer, W. Bar, J. Ballantyne, Selection of highly specific and sensitive mRNA biomarkers for the identification of blood, Forensic Sci. Int. Genet. 5 (2011) 449–458.
[15] J. Jakubowska, A. Maciejewska, R. Pawlowski, mRNA profiling in identification of biological fluids in forensic genetics, Probl. Forensic Sci. 87 (2011) 204–215.
[16] E. Hanson, C. Haas, R. Jucker, J. Ballantyne, Identification of skin in touch/contact forensic samples by messenger RNA profiling, Forensic Sci. Int. Genet. Suppl. Ser. 3 (2012) 305–306.
[17] S.M. Park, S.Y. Park, J.H. Kim, T.W. Kang, J.L. Park, K.M. Woo, J.S. Kim, H.C. Lee, S.Y. Kim, S.H. Lee, Genome-wide mRNA profiling and multiplex quantitative RT-PCR for forensic body fluid identification, Forensic Sci. Int. Genet. 7 (2013) 143–150.
[18] E. Hanson, C. Haas, R. Jucker, J. Ballantyne, Specific and sensitive mRNA biomarkers for the identification of skin in 'touch DNA' evidence, Forensic Sci. Int. Genet. 6 (2012) 548–558.
[19] E.K. Hanson, J. Ballantyne, Highly specific mRNA biomarkers for the identification of vaginal secretions in sexual assault investigations, Sci. Justice 53 (2013) 14–22.
[20] J. Jakubowska, A. Maciejewska, R. Pawlowski, K.P. Bielawski, mRNA profiling for vaginal fluid and menstrual blood identification, Forensic Sci. Int. Genet. 7 (2013) 272–278.
[21] A. Lindenbergh, M. van den Berge, R.J. Oostra, C. Cleypool, A. Bruggink, A. Kloosterman, T. Sijen, Development of a mRNA profiling multiplex for the inference of organ tissues, Int. J. Legal Med. 127 (2013) 891–900.
[22] D. van der Meer, M.L. Uchimoto, G. Williams, Simultaneous analysis of micro-RNA and DNA for determining the body fluid origin of DNA profiles, J. Forensic Sci. 58 (2013) 967–971.
[23] B. Bhoelai, B. de Jong, T. Sijen, Can mRNA markers distinguish traces generated by different types of contact? Forensic Sci. Int. Genet. Suppl. Ser. 4 (2013) e3–e4.
[24] C. Haas, E. Hanson, W. Bar, R. Banemann, A.M. Bento, A. Berti, E. Borges, C. Bouakaze, A. Carracedo, M. Carvalho, A. Choma, M. Dotsch, M. Duriancikova, P. Hoff-Olsen, C. Hohoff, P. Johansen, P.A. Lindenbergh, B. Loddenkotter, B. Ludes, O. Maronas, N. Morling, H. Niederstatter, W. Parson, G. Patel, C. Popielarz, E. Salata, P.M. Schneider, T. Sijen, B. Sviezena, L. Zatkalikova, J. Ballantyne, mRNA profiling for the identification of blood—results of a collaborative EDNAP exercise, Forensic Sci. Int. Genet 5 (2011) 21–26.

[25] C. Haas, E. Hanson, M.J. Anjos, W. Bar, R. Banemann, A. Berti, E. Borges, C. Bouakaze, A. Carracedo, M. Carvalho, V. Castella, A. Choma, G. De Cock, M. Dotsch, P. Hoff-Olsen, P. Johansen, F. Kohlmeier, P.A. Lindenbergh, B. Ludes, O. Maronas, D. Moore, M.L. Morerod, N. Morling, H. Niederstatter, F. Noel, W. Parson, G. Patel, C. Popielarz, E. Salata, P.M. Schneider, T. Sijen, B. Sviezena, M. Turanska, L. Zatkalikova, J. Ballantyne, RNA/DNA co-analysis from blood stains—results of a second collaborative EDNAP exercise, Forensic Sci. Int. Genet. 6 (2012) 70–80.

[26] C. Haas, E. Hanson, M.J. Anjos, R. Banemann, A. Berti, E. Borges, A. Carracedo, M. Carvalho, C. Courts, G. De Cock, M. Dotsch, S. Flynn, I. Gomes, C. Hollard, B. Hjort, P. Hoff- Olsen, K. Hribikova, A. Lindenbergh, B. Ludes, O. Maronas, N. McCallum, D. Moore, N. Morling, H. Niederstatter, F. Noel, W. Parson, C. Popielarz, C. Rapone, A.D. Roeder, Y. Ruiz, E. Sauer, P.M. Schneider, T. Sijen, D.S. Court, B. Sviezena, M. Turanska, A. Vidaki, L. Zatkalikova, J. Ballantyne, RNA/DNA co-analysis from human saliva and semen stains—results of a third collaborative EDNAP exercise, Forensic Sci. Int. Genet. 7 (2013) 230–239.

[27] C. Haas, E. Hanson, M.J. Anjos, K. Ballantyne, R. Banemann, B. Bhoelai, E. Borges, M. Carvalho, C. Courts, G. De Cock, K. Drobnic, M. Dötscr, R. Fleming, C. Franchix, I. Gomes, G. Hadzic, S.A. Harbison, J. Harteveld, B. Hjort, C. Hollard, P. Hoff-Olsen, C. Hüls, C. Keyser, O. Maroñas, N. McCallum, D. Moore, N. Morling, H. Niederstätter, F. Noël, W. Parson, C. Phillips, C. Popielarz, A.D. Roeder, L. Salvaderi, E. Sauer, P.M. Schneider, G. Shantan, D. Syndercombe Court, M. Turanská, R.A.H. van Oorschot, M. Vennemann, A. Vidaki, L. Zatkalíková, J. Ballantyne, RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains—results of a fourth and fifth collaborative EDNAP exercise, Forensic Sci. Int. Genet. 8 (2014) 203–212.

[28] J. Juusola, J. Ballantyne, Multiplex mRNA profiling for the identification of body fluids, Forensic Sci. Int. 152 (2005) 1–12.

[29] R.I. Fleming, S. Harbison, The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids, Forensic Sci. Int. Genet. 4 (2010) 244–256.

[30] A. Lindenbergh, M. de Pagter, G. Ramdayal, M. Visser, D. Zubakov, M. Kayser, T. Sijen, A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces, Forensic Sci. Int. Genet. 6 (2012) 565–577.

[31] A.D. Roeder, C. Haas, mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification, Int. J. Legal Med. (2012) 707–721.

[32] J. Harteveld, A. Lindenbergh, T. Sijen, RNA cell typing and DNA profiling of mixed samples; Can cell types and donors be associated, Sci. Justice 53 (2013) 261–269.

[33] C.C. Benschop, C.P. van der Beek, H.C. Meiland, A.G. van Gorp, A.A. Westen, T. Sijen, Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results, Forensic Sci. Int. Genet. 5 (2011) 316–328.